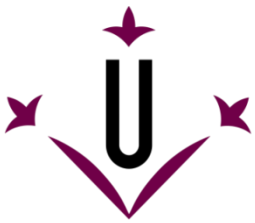

# Effect of Slurry Dilution, Structural Carbohydrates, and Exogenous Archaea Supply on *In Vitro* Anaerobe Fermentation and Methanogens Population of Swine Slurry

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**Abstract**

*Artificial slurry (4% dry matter) was prepared using fresh feces and urine obtained from four pregnant sows fed with commercial diet. Freeze-dried dairy cattle feces were used (external archaea) as co-inoculum (Co-i) and structural carbohydrates (CHO: apple pulp; sugar beet pulp; and wheat straw) as substrates. Bottles were incubated (39 6 1°C for 56 days), and the gas production was measured (mbar) and converted to the volume. A sample of produced gas was taken and analyzed for methane concentration using a gas chromatography. Bottles were opened at days 0, 25, and 56 to determine total bacteria, total, and hydrogenotrophic methanogens archaea (HMA) concentrations using quantitative polymerase chain reaction and population biodiversity using denaturing gradient gel electrophoresis (DGGE). Incubation time reduced the titers of total bacteria and archaea ($P < 0.01$) but did not modify HMA population. Doses of Co-i showed a positive correlation with HMA titers, although inter- acted with an incubation period ($P < 0.001$); at 5% Co- inoculation, total bacteria decreased significantly (0–25 days) but remained steady until day 56 ($P > 0.05$), whereas at 10% Co inoculation, titers decreased constantly. Most of the archaeal DGGE bands were observed in all samples, suggesting a common microbial population origin but Co-i sup- ply altered the DGGE structure of archaea populations.*

*Keywords: archaea, biodiversity, DGGE, methane, slurry*

## INTRODUCTION

The emission of methane ($CH_4$) and other greenhouse gases from livestock, their implications, and mitigation tech- nologies were the subjects of many studies in the recent years (i.e., [1]). Livestock emit $CH_4$ due to anaerobic organic matter (OM) fermentation processes occurring mostly in the rumen, and also in post-gastric compartments such as colon (horses, pigs) or cecum (rabbit). Moreover, as the anaerobe fermentation goes on, $CH_4$ synthesis may persist during manure storage (>30 days; [2]), especially relevant in the pit's slurry located under swine pens [3]. $CH_4$ emission in pigs is lower than in ruminants [4], and a significant fraction (around 50% of the enteric synthesis; [5]) comes from slurry storage. $CH_4$ emission from slurry should be considered with a double perspective, first environmental pollution, which highly depends on storage conditions (time, temperature, dilution, and pH [4]) and second, "methanization" of the slurry through its codigestion, which can be considered as a potential renewable energy source [$CH_4$] for industrial pur- poses [6,7]. In fact, the valorization of the produced biogas (approximately consists of 65% $CH_4$, 35% $CO_2$, and trace gases such as $H_2S$, $H_2$, and $N_2$) is energy saving and environ- mental friendly due to low emission of hazardous pollutants. In most cases, biogas can be valorized energetically in com- bined heat and power installation for the simultaneous gen- eration of heat and electricity [8].

$CH_4$ synthesis involves a synchronic OM fermentation by a consortium of microbial species. Initially, organic substrate is degraded to monomers that are fermented (by acidogenic bacteria) to volatile fatty acids (VFAs) and ketones among others. Throughout acetogenesis, products are transformed to hydrogen and acetic acid [9] and then hydrogenotrophic and methylotrophic methanogens transform them into $CH_4$ [10]. Both, methylotrophic and hydrogenotrophic methano- gens are essential in the whole process, but the knowledge of their roles over the storing process of slurry is rather lim- ited [11]. Also, enhancing the microbial activity by inoculating the slurry with an external source of microbes and supplying extra fermentable OM have been suggested as fea- sible strategies; however, understanding how the microbial consortium responds to such alteration has been scarcely studied.

This work aimed to study the $CH_4$ yield and incubation media characteristics (pH and VFA profile) as well as the abundance and biodiversity of methanogens during "*in vitro*"

anaerobic fermentation of swine slurry. In addition, we eval- uated the impact of dry matter (DM) dilution and the addition of agroindustrial by-products as an external source of structural carbohydrates and cattle's feces as microbial inoculants on incubation media and $CH_4$ production.

## MATERIALS AND METHODS

### Materials

*Inoculums*

The slurry was artificially prepared from fresh feces and urine to obtain 10% DM concentration. Excreta were obtained from four pregnant sows (Centre d' Estudis Porcins Torrelameu, Lleida, Spain) fed with commercial diet (DE: 3.124 Mcal / kg; crude protein [CP]: 14%). Feces collected by rectal extraction and urine by vulva massage. Feces and urine were homogenized, sampled (10%, for DM analysis), and retained (for 24 h at $4^{o}C$) until use.

*Co-inoculum*

Fresh feces from dairy cattle (considered as source of external methanogens) were used. They were collected fresh, then lyophilized, ground, and stored in sealed jars until use.

*Substrates (CHO)*

Commercial agricultural by-products (SBP, sugar beet pulp; AP, apple pulp, and WS, wheat straw) were chosen as substrates. By-products samples were dried ($60^{o}C$, 48 h), ground with hammer mill (1 mm) and stored in sealed jars until use. The proximate chemical analysis of inoculums and substrates is shown in Table 1.

**Experimental Development**

An *in vitro* anaerobic codigestion of slurry was conducted following [12] procedure and modified by [13]. One hundred and eight bottles were assigned to a 3 3 3 3 4 factorial arrangement that include three slurry dilutions (2, 4, and 6% DM), three Co-i concentrations (Co-i: 0, 5, and 10%), and three structural carbohydrates (CHO: SBP, AP, and WS) plus blank. Briefly, three mixtures were prepared to obtain 20:80, 40:60, and 60:40, slurry: buffer concentrations (2, 4, and 6% of DM concentration, respectively). Three levels of Co-i: 0, 5, and 10% (based on the slurry DM) were added to the artifi- cial slurry together with 600 g DM of the different CHO (SBP, AP, and WS). In addition, the corresponding blanks combinations were incubated along with the treatments as follows: (1) buffer 1 slurry; (2) buffer 1 slurry 1 Co-i (at 5 and 10% DM).

The bottles (160 mL volume) containing the media were incubated (39 6 1$^{o}$C) in quadruplicate for 56 days, and the measurements were repeated in two consecutive incubation batches. The incubation temperature for the *in vitro* digestion experiment was chosen based on the aforementioned protocols [12,13]. Although the temperature was rather high for the mesophilic bacteria but the inoculum's turn over and its pH value were efficient enough to evaluate anaerobic microbial fermentation by measuring gas production.

*Media Preparations*

Bottles were filled with their corresponding substrate and Co-i doses a day before the experiment began. Incubation solution was prepared following the initial protocol [12]. Then, the bottles were sealed with butyl rubber stopper and aluminum crimp seals in the presence of a constant $CO_2$ stream. The pressure was released using a hypodermic needle through the septum, after shaking the bottles and the incubation started in an air oven at 39 6 1$^{o}$C for 56 days.

*Sampling Protocol*

Gas production was measured from the headspace in mbar using a manometer (DELTA OHM, Caselle di Selvazzano, Italy) equipped with a probe (TP704). Gas measurements were performed daily (days 1–7), then twice a week (days 8–21), and once a week (day 22 to 56) at the end of the experiment. To prevent slight differences in total volume among bottles, the registered the head space pressure were converted to volume by a linear regression established between the recorded pressure in each bottle and known inoculated

air volumes at the same incubation temperature [12]. Gas volume at each incubation time was expressed per gram of incubated OM. After gas pressure measurements, a sample (0.1 mL) from head space gas was collected manually using Hamilton syringe, Gastight (1001SL1.0 mL SYR 22/2"/2 L, Hamilton Company, Reno, Nevada, USA) and immediately analyzed for $CH_4$ concentration. Incubation bottles were opened at days 0, 25, and the two left bottles at day 56, pH was immediately determined (pH meter 2000 Crucible, Crucible Instruments. Barcelona, Spain), and 12 mL of the liquid media were weighed, frozen in liquid nitrogen, and stored at 280$^{o}$C for molecular analyses. The remained contents were filtered through a metal sieve (1 mm Ø) and two samples were taken for $NH_3$-N (2 mL were acidified with 0.8 mL HCl 0.5 N) and VFAs determination (4 mL were mixed with preservative solution (1 mL of 2 g/L mercuric chloride, 20 mL/L of orthophosphoric acid, and 2 g/L of 4-methylvaleric acid in distilled water). Samples were immediately frozen (220$^{o}$ C) until further analyses.

*Chemical Analyses*

DM content was determined using air oven at 105$^{o}$C until a constant sample weight. Ash content was determined by incineration of the samples using muffle furnace (550$^{o}$C for 4 h). CP, crude fiber, and ether extracts were analyzed according to [14]. The $NH_3$-N concentration of the media was measured by distillation-titration based on the Kjeldahl methodology [14]. Neutral detergent fiber and acid detergent fiber were analyzed according to [15] procedures. VFA concentrations were determined by gas chromatography (GC) based on the technique proposed by [16] using a gas chro- matograph (Agilent Technologies 7890A, Net Work GC System, Beijing Elmer, Boston, USA), equipped with a flame ionization detector and capillary column (BP21 30m 3 0.25 mm ID 3 0.25 lm). $CH_4$ was analyzed using the same GC equipment, equipped with different column (113 4332, GS- Gaspro capillary 30m 3 0.32 mm ID), operating at 70$^{o}$C for the column, 150$^{o}$C at the injector, and 200$^{o}$C at the detector.

The carrier gas was helium (99.999% purity [C50], Carburos Metalicos, Spain) and the total injection time was 2 min. $CH_4$ concentration was calculated from the peak concentration: area ratio using as reference peak area generated from stand- ard gas ($CH_4$; 99.995% purity [C45], Carburos Metalicos, Spain). Then different head space volumes of the standard mixture (0.1, 0.3, 0.5, 0.7, and 0.9 mL) were manually injected into the gas

chromatograph to obtain the standard curve.

*DNA extraction, DGGE, and Real-Time PCR Analyses*

The DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) following the manufac- turer's instructions. For denaturing gradient gel electrophoresis (DGGE) analysis, specific Archaea region of the *16S* rRNA gene was amplified by polymerase chain reaction (PCR) using the primers described by [17]. PCR amplification condi- tions were as follows: 1 cycle (94$^{o}$C for 4 min); 30 cycles (94$^{o}$C for 1 min, 55$^{o}$C for 1 min, 72$^{o}$C for 1 min); 1 cycle (72$^{o}$C for 7 min).

**PCR Reactions**. DNA (50 ng) was used in a 50-mL reaction mix containing 1 mM buffer, 1.25 mM of each primer, 0.8 mM of dNTPs, 2.5 mM $MgCl_2$, and 2.5 U of Taq DNA polymerase in 10 mM Tris-HCl (pH 9.0). The resulting ampli- cons were visualized on a 2% (w/v) TBE (89 mM Tris, 89 mM Boric acid, 2mM $Na_2EDTA$, pH 8.3) agarose gel to check PCR products within the predicted size range. DGGE was performed using a V20-HCDC DGGE Unit (BDH) from VWR international Ltd (UK), following the manufacturer's guide-lines. PCR products (10 lL) were loaded onto 8% (w/v) TAE polyacrylamide gels (40 mM of Tris base, 20 mM of glacial acetic acid and 1 mM of EDTA, pH 8.3), which contained a 30–65% denaturant gradient (100% denaturant, 7 M of urea, and 40% (v/v) deionized formamide). Electrophoresis was performed at a constant voltage of 80 V and at a constant temperature of 60$^{o}$C for 16 h. Then DNA was visualized by silver stained using a Bio-Rad Silver Staining kit following the manufacturer's instructions. The gel was scanned and the image was analyzed with molecular analysis fingerprinting software (Quantity One - BIO-RAD Lab, Inc.) by scoring for the presence or absence of bands at different positions in each sample. DGGE banding profiles were compared using Dice coefficient and the Unweighed Pair Group Method with Arithmetic Mean (UPGMA) clustering algorithm and shown graphically as a dendogram. The level of similarity was indicated by the percentage similarity coefficient bar located above dendogram. Richness was estimated by counting the number of detected bands in each DGGE profile. The Shan- non index was used as a diversity index, as described in the following equation [18]: $H$ 5 2R ($pi$) (ln $pi$), where $pi$ is the ratio of one specific group of bacteria to the total microor- ganisms in the samples, and $i$ is the total number of micro- bial species in the samples. The evenness index was calculated as: H/ln total number of bands in the gel. Real- time PCR was used to quantify the numbers of total

bacteria [19] using the primer sets described in Table 2 as described previously by Abecia *et al*. [22]. Relative abundance of total methanogenic archaea (MA) and hydrogenotrophic methanogens were also determined using specific primers [21,23]. The relative quantification of both methanogen groups was carried out using D*C*t method (D*C*t 5 *C*t total bacteria – *C*t MA; [24]. Analyses were performed on iQ5 multicolor Real- time PCR Detection System (BioRad, Laboratories Inc., Her- cules, CA).

*Calculations and Statistical Analysis*

Pressure values (mbar) were transformed to the volume, by making standard curve using generated pressure against its known air volume, final equation was ($y$ 5 11.46$^{21}$ 3 20.289; $R^2$ 5 0.9984) being ($x$, mbar) and ($y$, mL) pressure and gas volume, respectively. Gas, $CH_4$ production, and pH were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC), considering the replicates (1, 2), slurry DM concentrations (2, 4, and 6%), types of substrate (blank, WS, AP, and SBP), Co-i levels (0, 5, and 10%), the incubation period (day 7, 14, 21, 28, 35, 42, 49, and 56), and their possi- ble interactions as fixed effects and the bottle as random effect.

Hence, there was no replication for VFA, $NH_3$-N, and quantitative PCR analyses and then the replication was not considered as fixed effect in their statistical analyses model. The copy numbers of total bacteria, total archaea, and hydro- genotrophic methanogens were transformed to their loga- rithm [log10] to perform the statistical analyses. The relative quantification of methanogens was carried out using D*C*t (D*C*t 5 *C*t total bacteria 2 *C*t MA). The mean separation between treatments was performed using the Tukey's test, and the differences were considered significant at $P < 0.05$.

## RESULTS AND DISCUSSION

### Methodological Approach

Pig slurry is a mixture of excreta, including feces and urine together with water and some feed orts [25] stored in the animal's pit and bulked temporally in the pool. Slurry hydration comes mostly from urine but also, at different pro- portions, from water refusals, animal's

cleaning or drinking losses. Moreover slurry storage periods are based on farm structure, management, and regulations imposed for their application in the field. Indeed, it results in a wide heterogeneity of slurry compositions and characteristics. As an example, slurry DM content (g DM/100 mL) in the Spanish commercial pig farms range from 1 to 12. Thus, in this work, we restricted the study on evolution of archaea titers during the anaerobic OM conversion to $CH_4$ at specific slurry dilutions varying from 2 to 6% of DM. Fresh pigs' feces were diluted with fresh urine up to 10% to simulate the normal feces/urine ratio and further diluted up to 6% (2, 4, and 6%) of DM using aforementioned buffer and tap water. To com- pensate the low C/N ratio of the slurry and thus to improve bacterial growth, media were supplemented with crop or agroindustrial by-products characterized by their high pro- portion of different type of structural carbohydrates. It was assumed that starch and other nonstructural sources were previously digested in the pig's intestine. The anaerobic digestion system used originated from the methodology pro- posed by Theodorou et al. [12] designed to optimize anaero- bic degradation of substrate at 39$^{o}$C, by buffering the pH variation and using small amounts of substrate. Under such experimental approach, this article was made and discussed.

**$CH_4$ Production and Incubation Media Characteristics**

Figure 1 shows the accumulated $CH_4$ production through- out the incubation period (from 0 to 56 days) at different substrate and slurry dilutions in the incubation media (Figure 1a and 1b, respectively). The $CH_4$ production was modified by slurry concentration ($P < 0.001$), although its effect was not linear, leveling off at 4% concentration when slurry was incubated without (53.42, 72.92, and 52.72 mL/g OM SE 5 6.53) or with (50.30, 70.21, and 55.84 mL/g OM SE 5 8.27 for slurry concentrations at 2, 4, and 6% of DM, respectively) substrate. Addition of WS and AP improved total $CH_4$ production (296 and 361 mL $CH_4$/bottle; $P < 0.05$) and the production efficiency (132.2 and 138.6 mL $CH_4$/ g OM; $P < 0.05$) regarding to blanks (251 mL $CH_4$/bottle and 124.18 mL $CH_4$/ g OM).

However, the addition of SBP depressed both total (211 mL $CH_4$/bottles) and production efficiency (88.48 mL $CH_4$/ g OM) compared with blanks. Co- inoculation with cows' feces did not affect $CH_4$ production.

$CH_4$ production at 56 days (mL/bottle) was directly related to the amount of available OM, but differences among treatments in relation to the production efficiency (mL/g OM

fermented) were relevant. $CH_4$ production (mL/g OM) was within the range reported by other authors which studied the similar conditions for both slurry concentration (4.3–13.6% DM) and incubation periods (up to 56 days) [26– 28], although higher production has been reported in some literatures (290–490 mL/g OM; [2]). Differences in efficiency of production must be interpreted in terms of variation in the fermentation conditions and initial substrate [29].

During the incubation period, pH showed the pattern showed in Figure 2, which decreased at 25-days and increased at 56-days period ($P < 0.001$), although the variation was more pronounced in supplemented than in nonsupplemented media (blanks) in which pH reduction at 25-days period was negligible (interaction days 3 substrate: $P < 0.01$). A differential evolution among the substrates was observed with slurry dilution as pH decreased in SBP-supplemented bottles with 6% slurry concentration (6.9, 6.79, and 6.67). The opposite was observed in nonsupplemented bottles (blanks: 7.07, 7.27, and 7.33 for 2, 4, and 6% slurry DM concentration), whereas no significant variations were detected in those bottles supplemented with WS or AP (inter-action slurry dilution 3 substrate: $P < 0.01$). Type of sub- strate altered average media acidity ($P < 0.01$) and pH was lower in the pulps (pH 57.02 and 6.8 for AP and SBP, respectively) than in blanks bottles (7.23; SE: 0.04).

Ammonia-N concentration in the incubation media varied between 1.18 to 3.02 g/L and it was significantly modified by experimental treatment. Ammonia concentration increased throughout the incubation period in the highest slurry con- centration (2.57, 2.72, and 3.02 g/L in 6% DM slurry at 0, 25, and 56 days compared with 1.58, 1.92, and 2.11 g/L at 4% DM slurry, respectively). However, no changes were observed at the lowest concentration level of the slurry (1.56, 1.18, and 1.22 in 2% DM slurry at 0, 25, and 56 days incubation period, respectively; interaction slurry concentration 3 days: $P > 0.01$). The effect of slurry concentration on ammonia was also modulated by type of substrate; thus, for SBP-supplemented bottles and for blanks bottles, ammonia concentration increased proportionally with DM concentra- tion, but in those bottles supplemented with WS or AP, the increase was only observed when slurry concentration changed from 4 to 6% of DM.

$CH_4$ production did not increase with the highest slurry DM concentration. Thereby, it was found that there was not a direct relationship between the OM concentration in the media and $CH_4$ production efficiency, which agrees with pre- vious reports [30,31]. In this sense, it has been hypothesized that the absence of a direct relationship could be derived either

from the presence of toxic substances in the slurry (i.e., lignin and its derivatives: [18,32] or because the incuba- tion media became inappropriate or toxic, for the acidity induced by the excess of VFAs [32] or on the contrarily, alka- linity induced by high ammonia loads [18]. The average of VFAs concentration (mM) in the original media (12.7) was increased (28.8) at day 25 and then decreased (6.1) at day 56 of the incubation period (Tables 3 and 4). The differences among VFA concentration of different slurries during the incubation course did reach statistical significance (interaction; slurry concentration 3 days: $P > 0.05$). The highest production (from day 0 to 25) was registered for the highest DM concentration (6%), whereas the highest disappearance rate (from day 25 to 56) was observed with the medium slurry concentration (4% DM). Changes in the molar proportion of main individual VFAs (i.e, acetic, propionic, and butyric) are shown in Figure 3. In general, acetate proportion increased at different rates, to the detriment of the rest, although such transformation pro- cess was modulated in both, slurry concentration and type of substrate.

During the incubation period, in the most diluted slurry, the average concentration of acetate was greater while the concentration of propionate was lower in the blank and WS than in AP and SBP (2% DM slurry; $P < 0.05$). This trend was not observed in 4 and 6% DM slurry, which showed similar average concentration of acetate and propionate among sub- strates ($P > 0.05$). As it is shown in Figure 3, the relative production of propionate was quite negligible at day 56 of incubation in all the substrates in 4 and 6% DM slurry but it was noticeable for AP and SBP in the 2% DM slurry. The concentration of valerate and branched-chain fatty acids (iso-butyrate plus iso-valerate acids) was affected by the incuba- tion period where the interaction of slurry dilution 3 incubation time were significant ($P < 0.01$; Tables 3 and 4). Valerate proportion peaked at day 25 in 4 and 6% DM (2.55 and 2.02 6 0.16%, respectively), whereas this increase was not observed in 2% DM slurry (1.51 6 0.16%, $P < 0.05$). Similarly, the proportion of branched-chain fatty acids peaked at 25 days of incubation period in 4 and 6% DM slurry (6.62 and 6 0.56%, respectively), but not in 2% DM slurry (3.40 6 0.56%, $P < 0.05$). In addition, valerate and branched- chain fatty acids concentrations were negligible at 56 days of incubation.

The high buffer capability of the slurry was able to com- pensate induced acidity by increases in VFA production in the case of blanks and bottles supplemented with WS, as pH level was kept within the appropriate range reported for anaerobic OM digestion (6.6–7.6; [33]). However, pH was below this threshold level and reached a critical value in those media supplemented with pulps, which explains the inhibition of the fermentation processes and the registered depression in $CH_4$ production using pulps [34]. SBP is quickly fermented in the initial digestion process (days 1–10; [35] due to its high proportion of high digestible hemicellulo- ses and low proportion of lignin and cellulose [36], which yields high production/accumulation of VFA [34] and the acidification of the media may temporally depress microbial fermentation [37,38]. Few results are available in relation to AP digestion, but registered values on pear pulp [39] were similar to those in this study. In any case, the changes in the pH cannot justify the depression in $CH_4$ production regis- tered at the highest level of slurry concentration.

Slurry as substrate for microbial growth has two main constraints: the low availability of OM together with high concentration of N mostly as ammonium form. $NH_3$-N concentrations in the blanks are within the normal values reported in slurries from commercial farms [3,25]. Ammo- nium nitrogen comes from urea hydrolysis (carried out in ubiquitous microbial urease [40] and it may explain the varia- tions in $NH_3$-N concentration regarding to the original slurry (blanks)). The evolution of $NH_3$-N concentration during the incubation period was altered by the experimental treatment and it may reflect the balance between microbial protein degradation and synthesis [41]. Microbial protein synthesis may prevail over degradation processes in those media regarding the lowest DM concentration slurry, therefore decreasing the $NH_3$-N concentration, whereas protein degra- dation may predominate as slurry concentration increases. The unbalanced $NH_3$-N increased with carbohydrate supple- mentation, basically due to addition of pulps. Hansen *et al*. [34] reported that $NH_3$-N would exert inhibition when values reach 3.3 g/L, whereas Chen et al. [17] increased such thresh- old level up to 5 g $NH_3$-N per liter by a previous media adaptation to the substrate. More specifically Van Velsen [42] and Hashimoto [43] described a depression in titers of metha- nogen archaea when $NH_3$-N reached values up to 1.5–2.5 g/
L. That was our case, and the negative effect of $NH_3$-N level on microbial growth with the highest concentration of slurry could not be discarded.

The anaerobic decomposition of biowaste occurs in continuous process, although specific phases could be distinguished [9]; in our case, bottles were sampled at discrete

timing. At 0 days, VFA concentrations reflect values of the original slurry. In the adult pig's intestine, most of starch and other nonstructural carbohydrates are digested and absorbed, so those remaining in the feces belong mostly to structural carbohydrates group (cellulose and other fibrous products) and thus acetic acid was the most predominant VFA [44,45]. At 25 days, a significant increase in VFA concentrations was observed. Media reflected the initial stage of the syntrophic degradation characterized by hydrolysis products and VFA synthesis [38]. Finally, at 56 days, there was a significant disappearance of VFA that led to acetic acid prevalence. This trend would reflect the transformation of short fatty acid and other product to acetic acid by acetogenic bacteria and further degradation to $CH_4$ by methanogens [9].

Slurry dilution and pulp addition increased propionic acid prevalence at 56 days sampling. In this sense, we hypothe- sized that non-acetic VFA prevalence may be explained by two reasons, the excess of substrate which delayed the syntrophic degradation process or increase in pulp supplementation [46,47], which promoted the concentration of reducing population [17] and they may compete against methanogens for substrate [49].

The greatest branched-chain VFA concentrations at 25 days incubation in the most concentrated slurries (4 and 6% DM) was in accordance with the increase in $NH_3$-N concentration derived from protein degradation and subsequently availability of branched-chain amino acids, which are neces- sary for iso-fatty acids synthesis [50]. However, the accumulation of branched-chain VFA was not sustained at 56 days of incubation even though $NH_3$-N kept increasing.

**Evolution of Total Bacteria and Archaea Numbers Throughout Incubation Period**

Methanogens are sensitive to changes in their niche, as their habitat is limited to strict conditions and their substrates are generally limited [51]. The physicochemical conditions, bacterial community composition and OM are the keys of environmental factors that allow methanogens to survive in both *in vitro* anaerobic digestion and natural ecosystems (i.e., marshes, riverbed sediments, and lake sediments [52]). In fresh slurries, several archaea communities were described [53], being such communities dominated by hydrogenotro- phic and methylotrophic microorganisms that are able to adapt and survive in *in vitro* conditions. However, ammonia [31] and the excess of organic load [54] were identified as the common limiting factors. Figure 3 shows that the intermediate slurry concentration (4% of DM) at 56-day period pro- vided the most

favorable fermentation conditions to allow the complete OM fermentation. Most of the substrate was fermented to $CO_2$ and $CH_4$; at this point, the complete trans- formation or methanization of the fermentable substrate may be assumed. This was not seen either in highest or lowest slurry concentration medias. For these reasons, the profile and evolution of total bacteria and archaea populations were only analyzed in 4% DM slurry bottles and corresponding data are presented in Table 5 and 6. The addition of different types of substrate did not alter the total bacteria concentra- tion in the bottles comparing with the blanks. However, total bacterial biomass decreased significantly throughout the incubation period (from 15.1 to 3.1 log gene copy numbers 16S-rRNA/g FM, Table 5 and 6). An interaction of Co-i addition level with incubation day was seen in the total bacteria gene copy numbers ($P < 0.001$). In 5% Co-i level, total bacte- ria decreased significantly from day 0 to 25 (14.8 vs. 4.9, $P < 0.05$) but remained steady until 56 incubation days (3.9, $P > 0.05$), while in 10% Co-i level the early evolution was similar (9.4 vs. 5.1, $P < 0.05$) but the late evolution was lower (3.4, $P < 0.05$). The relative abundance of total archaea was also depressed throughout the incubation time ($P < 0.01$), independent of substrate and Co-i supply. On the contrary, in total archaea population, hydrogenotrophic methanogens population showed an increase in the relative abundance at day 25 and then they declined from days 25 to 56. Also their titers increased proportional with Co-i presence in the media (Table 5 and 6, $P < 0.05$). Moreover, hydrogenotrophic methanogens proportion decreased in those bottles supplemented with WS ($P < 0.05$).

The quantification of total archaea population (targeting 16S rRNA gene) and the hydrogenotrophic group (targeting the methyl-CoenzM reductase (mcr*A*)) allowed us to monitor the patterns of the two main methanogenic groups described in this environment. This was performed assuming that the difference between total and hydrogenotrophic would corre- spond to acetotrophic methanogens. It is true that conditions imposed by an *in vitro* system are more uniform than natural ecosystems [55]; however, in *in vitro* cultivation and slurry maturation, both total bacterial and archaeal biomass were reduced.

In accordance with previous reports, hydrogenotrophic methanogens constituted a minor proportion within the archaea group in the slurry, which suggests that the major group corresponded to methylotrophic methanogens [56].

In our study, as the *in vitro* incubation progressed, a decrease in the abundance of archaea titers followed by a pronounced decrease of hydrogenotrophic group was observed. In this sense, [53] reported signifi changes in methanogens population throughout slurry maturation period due to a differential capability and effi in the adaptation process. Addition of freeze dry cow's feces to the incubation media improved titers of total archaea and hydrogenotropic methanogens. Nevertheless, the improve- ment in hydrogenotrophic titers did not lead to higher $CH_4$ production although $CH_4$ production efficiency increased.

**Biodiversity, DGGE Analyses**

The structure of the archaeal community in samples from swine feces (inoculums), and after anaerobic fermentation including different substrates and cows feces (Co-i) were analyzed by DGGE (Figure 4).The amplification of DNA using the set of archaeal primers (Table 2) generated 38 major different bands, 25 in the original feces (B0). Overall, samples shared 59% similarity in the banding profile and clear clustering pattern by incubation time: samples from day 56 clustered separately from those at days 0 and 25. Within another cluster, samples from days 0 and 25 also resulted in a different profile. Moreover, at the long time incubation period (56 days) the differences induced by Co-i addition were reflected in their community structure and those sam- ples supplemented either 5 or 10% of Co-i DM, clustered separately. In the latter case (addition of 10% of Co-i DM) the number of bands increased which was reflected in higher diversity. Indeed, in the original samples without (B0) or with Co-i (B5% 5 25 and B10% 5 25 bands) at 0-day incuba- tion period, the number of bands increased with Co-i addi- tion (addition of 10% of Co-i DM) although differences were more pronounced at 25 days of incubation period (25, 25.5, and 29.5 bands) than at 56 days (25, 25.5, and 27.5 band corresponding to Co-i addition of 0, 5, and 10% of DM, respectively; interaction day 3 Co-i reached statistical significance [$P < 0.05$]). Shannon and evenness indices showed a similar pattern to band numbers. Hence, using cows' feces coinoculum (Co-i) improved biodiversity throughout the incubation period. The predominance of hydrogenothopic methanogens in the ruminant digestive system [57] may explain the amelioration in the hydrogenotrophic population titers into the cultivation media and their slow adaptation capability to the new *in vitro* environment [58]. The optimization of the use of other sources of methanogens appears as an interesting area of work.

## CONCLUSIONS

The dilutions of the slurry (DM content) is the major fac- tor affected *in vitro* $CH_4$ production, while the addition of the three sources of external fermentable carbohydrates (WS, AP, and SBP) or cow feces did not seem to improve overall $CH_4$ production. The archaeal community structure adapts throughout the incubation period and was highly influenced by the addition of cows' feces, which resulted in higher $CH_4$ productions rates. Prospect of the microbial inoculums to optimize $CH_4$ production deserves further research.


## ACKNOWLEDGEMENT

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## ABBREVIATIONS

| | |
|---|---|
| AP | apple pulp |
| $CH_4$ | methane |
| CHO | structural carbohydrates |
| Co-in | co-inoculum |
| CP | crude protein |
| DGGE | denaturing gradient gel electrophoresis |
| DM | dry matter |
| GC | gas chromatography |
| HMA | hydrogenotrophic methanogens archaea |
| MA | methanogenic archaea |
| OM | organic matter |
| SBP | sugar beet pulp |
| VFA | volatile fatty acids |
| WS | wheat straw |

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**Table 1. Chemical composition of the inoculum (swine and cattle feces) and substrates (g/100 g DM).**

| | Inoculum | | Substrates | | |
|---|---|---|---|---|---|
| | Swine | Cattle | SBP | AP | WS |
| OM | 77.73 | 90.14 | 95.36 | 97.31 | 96.15 |
| Ether extract | 2.22 | 2.27 | 0.69 | 3.71 | 0.99 |
| CP | 15.46 | 17.84 | 10.4 | 9.59 | 3.04 |
| Crude fiber | 20.16 | 29.23 | 20.1 | 35.36 | 43.59 |
| Neutral detergent fiber | 45.05 | 46.38 | 40.22 | 58.58 | 82.5 |
| Acid detergent fiber | 21.18 | 26.22 | 23.77 | 40.72 | 53.63 |

SBP, sugar beet pulp; AP, apple pulp; WS, wheat straw.

**Table 2.** Authors and sequences of primers used in this experiment.

| | | Primers | |
|---|---|---|---|
| **Target** | **Author** | **Forward** | **Reverse** |
| DGGE Archaeae | [20] | 5′-CAGCCGCCGCGGTAA-3′ | 5′-CGCCCGCCGCGCCCCGCGCCCGGCCGC CGCCCCCGCCCCGTGCTCCCCCGCCAATTCCT-3′ |
| Quantitative PCR | | | |
| Total bacteria | [19] | 5′-GTGSTGCAYGGYTGTCGTCA-3′ | 5′-ACGTCRTCCMCACCTTCCCC-3′ |
| Total Archaeae | [21] | 5′- AGGAATTGGCGGGGGAGCA-3′ | 5′- BGGGTCTCGCTCGTTRCC-3′ |
| Hydrogenotrophicphic methanogens | [21] | 5′-TTCGGTGGATCDCARAGRGC −3′ | 5′-GBARGTCGWAWCCGTAGAATCC-3′ |

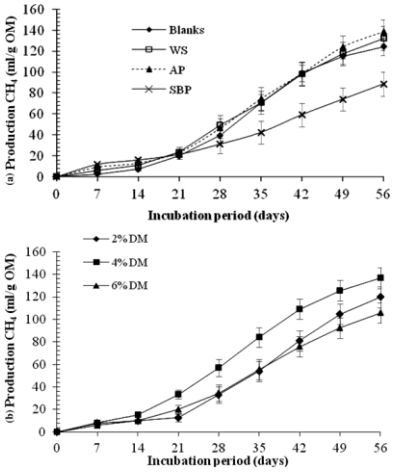


**Figure 1.** $CH_4$ production (milliliter per gram of OM) throughout the incubation period as affected by substrate addition (a) and slurry concentration (b).

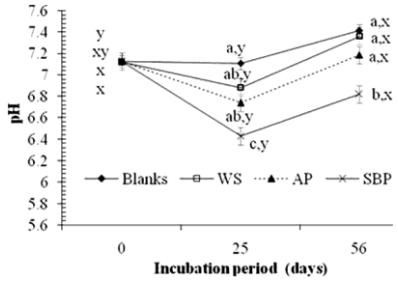


**Figure 2**. Average of pH values after adding structural car- bohydrates substrates (Blanks; WS: wheat straw; AP: apple pulp; SBP: sugar beet pulp) throughout the incubation times at 0, 25, and 56 days of incubation. Superscript letters a,b,c denote statistical differences ($P < 0.05$) among substrates within the incubation period, whereas x,y denote statistical differences ($P < 0.05$) within each substrate throughout the incubation period.

**Table 3.** Media fermentation parameters (pH, $NH_3$-N, and VFA) as affected by slurry concentration (2, 4, and 6 g DM/100 mL), incubation time (0, 25, and 56 days), and substrate supplementation (600 mg DM/bottle; WS, AP, or SBP).

| | Slurry DM concentration (%) | | | | Substrate | | | | | Days | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | 2 | 4 | 6 | SEM | Blank | WS | AP | SBP | SEM | 0 | 25 | 56 | SEM |
| pH | 7.0 | 7.2 | 7.3 | 0.04 | 7.3 | 7.2 | 7.1 | 7.1 | 0.04 | 7.4 | 6.9 | 7.2 | *0.04* |
| $NH_3$-N (g/L) | 1.6 | 2.3 | 3.4 | 0.07 | 2.4 | 2. 4 | 2.4 | 2.4 | 0.08 | 2.3 | 2.4 | 2.6 | *0.07* |
| VFA (mM) | 15.0 | 12.8 | 19.8 | 1.34 | 15.1 | 13.9 | 17.1 | 17.4 | 1.55 | 12.7 | 28.8 | 6.1 | *1.34* |
| VFA (mol/100 mol) | | | | | | | | | | | | | |
| Acetate | 63.1 | 73.8 | 72. 9 | 2.53 | 76.9 | 74.4 | 67.1 | 61.3 | 2.91 | 64.1 | 64.6 | 81.1 | *2.53* |
| Propionate | 27.0 | 16.8 | 16.4 | 2.27 | 14.5 | 16.0 | 23.0 | 26.7 | 2.61 | 23.5 | 19.2 | 17.6 | *2.27* |
| Butyrate | 4.7 | 4.7 | 5.3 | 0.28 | 3.8 | 4.6 | 5.2 | 5.9 | 0.32 | 5.9 | 8.8 | 0.04 | *0.28* |
| Valerate | 1.0 | 1.3 | 1.1 | 0.09 | 1.1 | 1.1 | 1.2 | 1.3 | 0.12 | 1.4 | 2.1 | 0.02 | *0.09* |
| BCFA | 4.2 | 3.5 | 4.3 | 0.37 | 3.7 | 3.9 | 3.4 | 4.8 | 0.44 | 5.2 | 5.4 | 1.2 | *0.37* |

BCFA, branched-chain volatile fatty acids; SBP, sugar beet pulp; AP, apple pulp; WS, wheat straw.

**Table 4.** Media fermentation parameters (pH, $NH_3$-N, and VFA) as affected by slurry concentration (2, 4, and 6 g DM/100 mL), incubation time (0, 25, and 56 days), and substrate supplementation (600 mg DM/bottle; WS, AP, or SBP).

| Item | Slurry | Substrate | Day | Slurry 3 substrate | Slurry 3 day | Substrate 3 day |
|---|---|---|---|---|---|---|
| pH | ** | ** | ** | NS | * | ** |
| $NH_3$AN (g/L) | ** | NS | * | ** | ** | NS |
| VFA (mM) | ** | NS | ** | NS | ** | NS |
| VFA (mol/100 mol) | | | | | | |
| Acetate | ** | ** | ** | * | ** | NS |
| Propionate | ** | ** | NS | * | ** | * |
| Butyrate | NS | ** | ** | NS | ** | ** |
| Valerate | NS | NS | ** | NS | ** | NS |
| BCFA | NS | NS | ** | NS | ** | NS |

Ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$. BCFA, branched-chain volatile fatty acids.

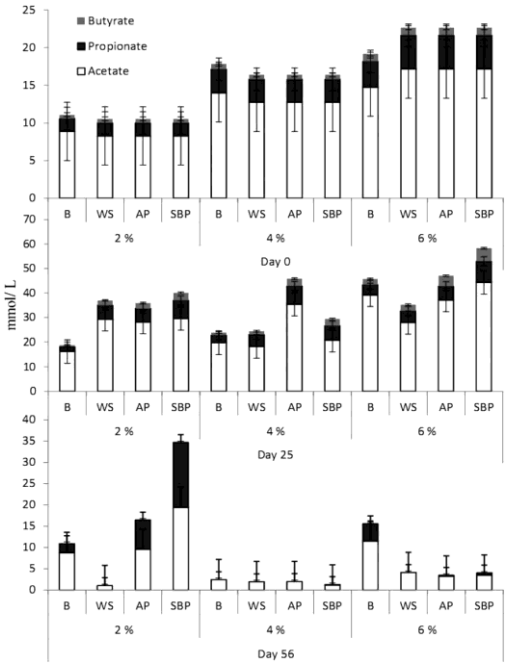


**Figure 3**. Concentration (millimoles per liter) of acetic acid, propionic acid, and butyric acid in bottles (media) containing swine slurry as inoculum (at 2, 4, or 6 gDM/100 g FM) at different incubation periods (0, 25, and 56 days) and supplemented with different levels of Co-i (0, 5, and 10 g/100 g DM lyophilized cows' feces) and crop by-products as substrates (B, blank; WS: wheat straw; AP: apple pup; SBP: sugar beet pulp).

**Table 5.** Effect of substrate supplementation (600 mg DM/bottle; WS, AP, or SBP), incubation time (0, 25, and 56 days), and Co-i addition (0, 5, or 10% of bottle DM) on $CH_4$ production (milliliters per bottle per day) and concentration of total bacteria (gene molecules 16S-rRNA/g FM), MAs (percentage of total bacteria; DC(t)), and hydrogenotrophic methanogens (percentage of total methanogens; DC(t)) in 4% DM slurry.

| | Substrate | | | | | Days | | | | Co-i (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | Blank | WS | AP | SBP | SEM | 0 | 25 | 56 | SEM | 0 | 5 | 10 | SEM |
| $CH_4$ production | 48.8 | 53.8 | 47.2 | 48.7 | | 0 | 46.5 | 102.4 | | 32.6 | 55.9 | 60.4 | |
| Total bacteria | 7.8 | 7.7 | 7.5 | 7.4 | 0.36 | 15.1 | 4.7 | 3.1 | 0.38 | 9.0 | 7.9 | 6.0 | 0.56 |
| Metanogenic archaea | 0.4 | 0.3 | 0.3 | 0.3 | 0.05 | 0.5 | 0.3 | 0.1 | 0.05 | 0.2 | 0.4 | 0.4 | 0.43 |
| Hydrogenotrophic methanogens | 0.5 | 0.1 | 0.6 | 0.3 | 0.16 | 0.1 | 0.6 | 0.3 | 0.17 | 0.0 | 0.3 | 0.7 | 0.16 |
| Biodiversity | | | | | | | | | | | | | |
| Bands number | 25.3 | 25.5 | 24.2 | 25.0 | 0.50 | 22.7 | 26.5 | 25.8 | 0.53 | 24.7 | 24.3 | 26.0 | 0.48 |
| Shannon index | 3.2 | 3.2 | 3.2 | 3.2 | 0.02 | 3.1 | 3.3 | 3.3 | 0.02 | 3.2 | 3.2 | 3.2 | 0.02 |
| Evenness | 0.8 | 0.8 | 0.8 | 0.8 | 0.005 | 0.8 | 0.8 | 0.8 | 0.005 | 0.8 | 0.8 | 0.8 | 0.004 |

**Table 6.** Effect of substrate supplementation (600 mg DM/bottle; WS, AP, or SBP), incubation time (0, 25, and 56 days), and Co-i addition (0, 5, or 10% of bottle DM) on $CH_4$ production (milliliters per bottle per day) and concentration of total bacteria (gene molecules 16S-rRNA/g FM), MAs (percentage of total bacteria; DC(t)), and hydrogenotrophic methanogens (percentage of total methanogens; DC(t)) in 4% DM slurry.

| *P* Value | Substrate | Days | Co-i | Co-i 3 day | Substrate 3 day |
|---|---|---|---|---|---|
| $CH_4$ production | Ns | *** | * | Ns | Ns |
| Total bacteria | Ns | ** | ** | ** | Ns |
| Methanogenic archae | Ns | ** | Ns | Ns | Ns |
| Hydrogenotrophic methanogens | * | Ns | * | Ns | Ns |
| Biodiversity | | | | | |
| Bands number | Ns | ** | ** | ** | Ns |
| Shannon index | Ns | ** | * | ** | Ns |
| Evenness | Ns | *** | * | ** | Ns |

Ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

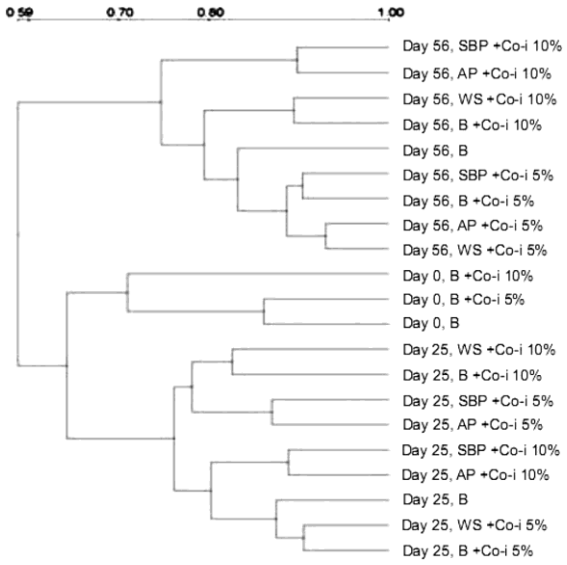


**Figure 4**. DGGE-derived dendrograms illustrating the effect of incubation period (0, 25, and 56 days), Co-i (0, 5, and 10% additions of lyophilized cows' feces) and substrate (B, blank; WS: wheat straw; AP: apple pulp; SBP: sugar beet pulp) on the structure of the archaeal community. The scale bars show Hamming distances.